Arch Virol (2007) 152: 1531–1539
DOI 10.1007/s00705-007-0979-0
Printed in The Netherlands

Archives of Virology

Brief Report

Homologous recombination plays minor role in excision of unit-length viral genomes from head-to-tail direct tandem repeats of porcine circovirus during DNA replication in *Escherichia coli*

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Received February 15, 2007; accepted April 2, 2007; published online May 14, 2007

Summary

In this report, we confirmed previous work that a theta-replicating bacterial plasmid containing 1.75 copies of genomic porcine circovirus (PCV) DNA in head-to-tail tandem (HTT) [a partial copy of PCV type 1 (PCV1), a complete copy of PCV type 2 (PCV2) and two origins of DNA replication (Ori)] yielded three different double-stranded DNA species when transformed into *Escherichia coli*: the input construct (*U*), the unit-length PCV1/PCV2 genome with a composite Ori lacking the plasmid vector (Q_{RC}) and the remaining left-over 0.75 copy PCV1/PCV2 genome with a different composite Ori inserted in the plasmid vector (L_{RC}). Replication of *U* was presumably via the theta-like replication mechanism utilizing the colicin E_1 Ori, while derivation of L_{RC} and Q_{RC} was via the rolling-circle replication (RCR) copy-release mechanism and required the presence of two PCV Oris and the virus-encoded Rep protein. Essentially, excision of a unit-length PCV1/PCV2 genome (Q_{RC}) via RCR from *U* yielded L_{RC}. Furthermore, we examined whether homologous recombination may also result in excision of a different type of unit-length PCV genome (Q_H) from identical HTT constructs to generate L_H. Whereas the generation of L_{RC} is Rep-protein-dependent, the generation of L_H is Rep-protein-independent. Accordingly, the L_{RC} and Q_{RC} molecules derived from RCR possess different characteristics from the L_H and Q_H molecules generated via homologous recombination. In one of the studies in which both L_{RC} and L_H were generated simultaneously from the same HTT construct, out of 69 samples analyzed, 66 were derived via RCR and 3 were derived via homologous recombination. Thus, in comparison with RCR, homologous recombination plays a minor role in the excision of unit-length PCV genomes from HTT constructs in *Escherichia coli*.

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Porcine circovirus (PCV) is a member of the genus *Circovirus* of the family *Circoviridae*, which

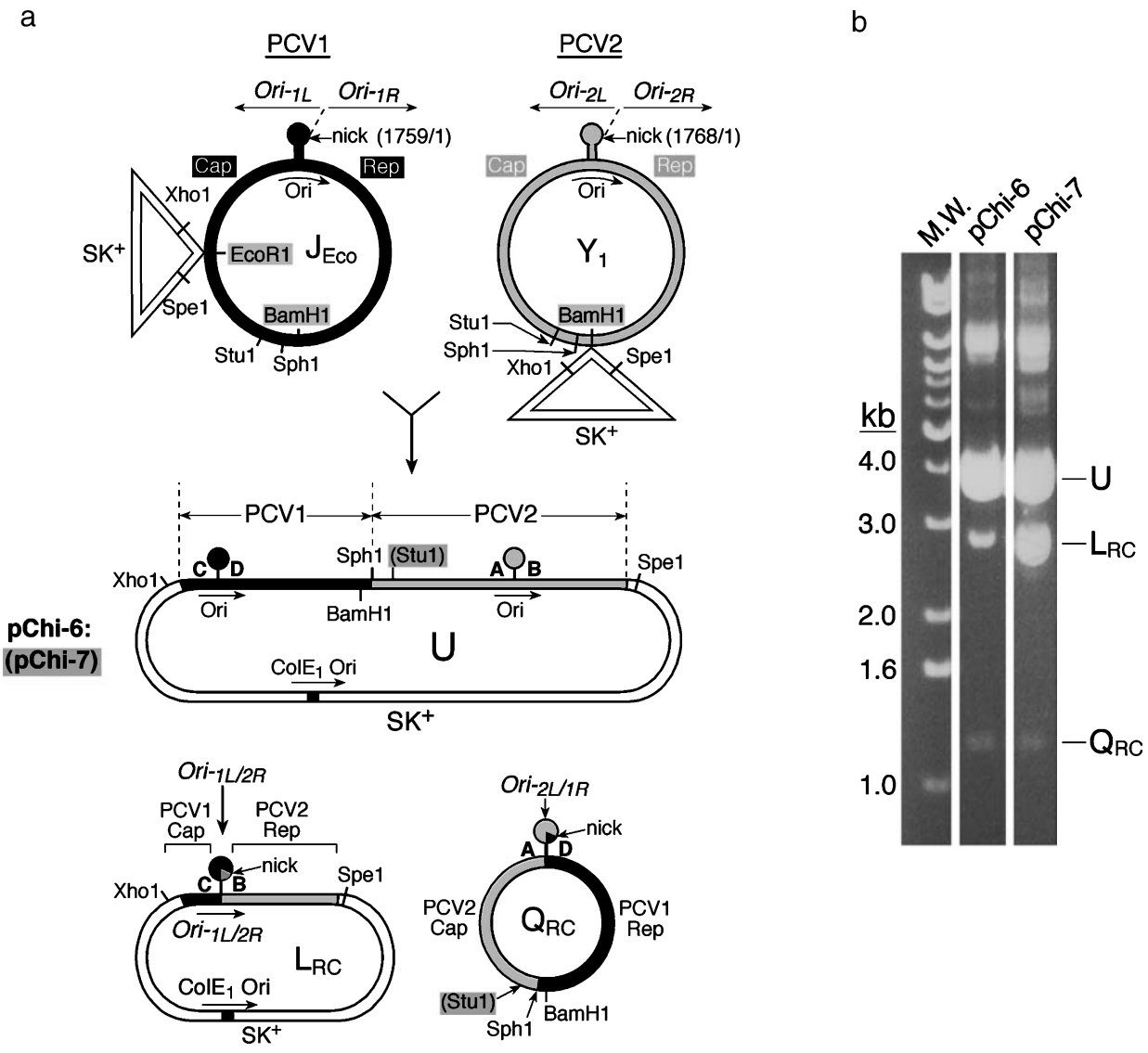


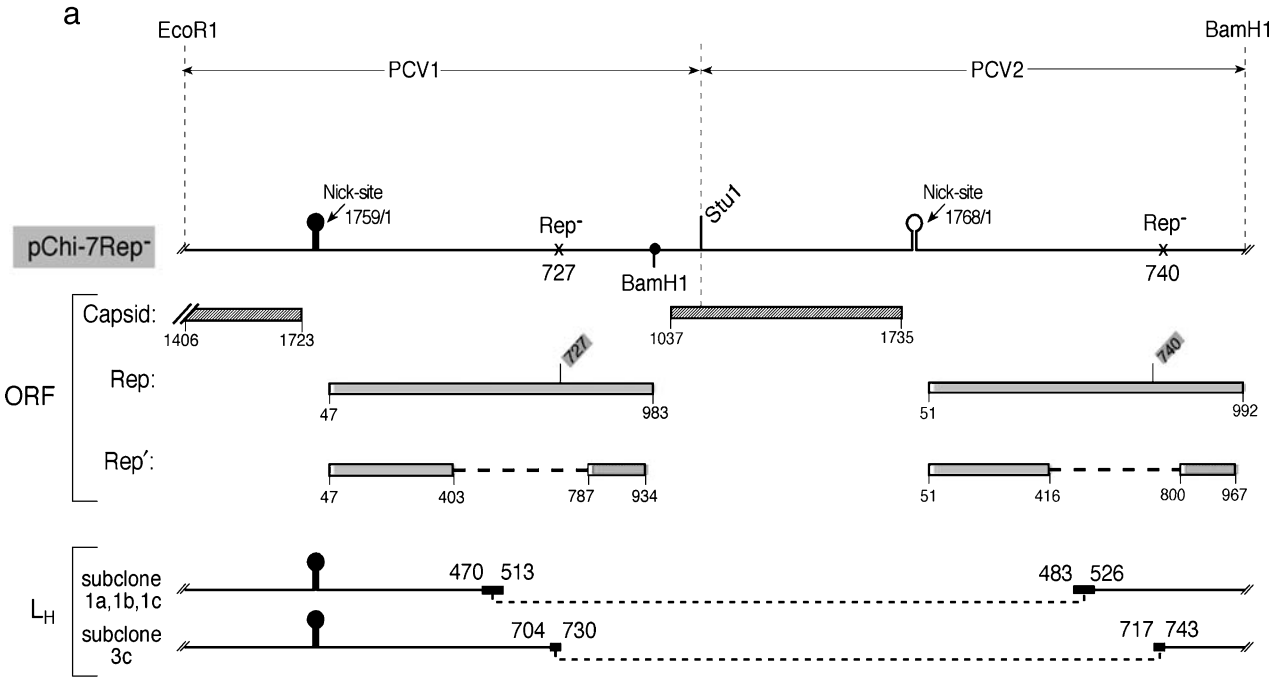
Fig. 1. a Schematic representation of pChi-6 and pChi-7 with their respective *U*, L_{RC} and Q_{RC} DNA species. A PCV1 genomic clone (J_{Eco} with two engineered sites, EcoR1 at nt 1406 and BamH1 at nt 992) (GenBank accession number AY184287) and a PCV2 genomic clone (Y_1 with an engineered BamH1 site at nt 1015–1020) (GenBank accession number AY094619) inserted into the pSK^+ plasmid (Stratagene, La Jolla, CA) were used to generate two chimeric head-to-tail tandem constructs containing 0.8 copy of PCV1 and 0.95 copy of PCV2. To generate pChi-6 or pChi-7, the Sph1-Spe1 or Stu1-Spe1 fragment containing the PCV2 Ori of Y_1 was swapped with the small Sph1-Spe1 or Stu1-Spe1 DNA fragment (not containing the PCV1 Ori of J_{Eco}), respectively. PCV1 DNA is indicated in black, PCV2 DNA is shaded, and pSK^+ DNA is in an open box. The Cap and Rep genes are located on the left and right side of the Ori, respectively. The left side ($-_{1L}$ and $-_{2L}$) and right side ($-_{1R}$ and $-_{2R}$) of the Ori are labeled with subscripts. The restriction enzyme sites relevant to this study are denoted, and entries specific to pChi-7 are in shaded parentheses. The Xho1 site is present only in the pSK^+ sequence and the Stu1 site is present in both the PCV1 and PCV2 genome. The BamH1 and Sph1 sites were engineered into the constructs for cloning purposes. The genomic sequence lengths of PCV1 (1759 nt) and PCV2 (1768 nt) are indicated. The nick site (indicated by ↓) is present within the octanucleotide (AGTATT↓AC). The stem-loop positions of PCV1 (positions C and D) and PCV2 (positions A and B) are also indicated. **b** Agarose gel electrophoresis of plasmid DNA recovered from *E. coli* TOP10 cells transformed with pChi-6 or pChi-7. The predominant closed-circular double-stranded molecules are labeled *U*, L_{RC} and Q_{RC}

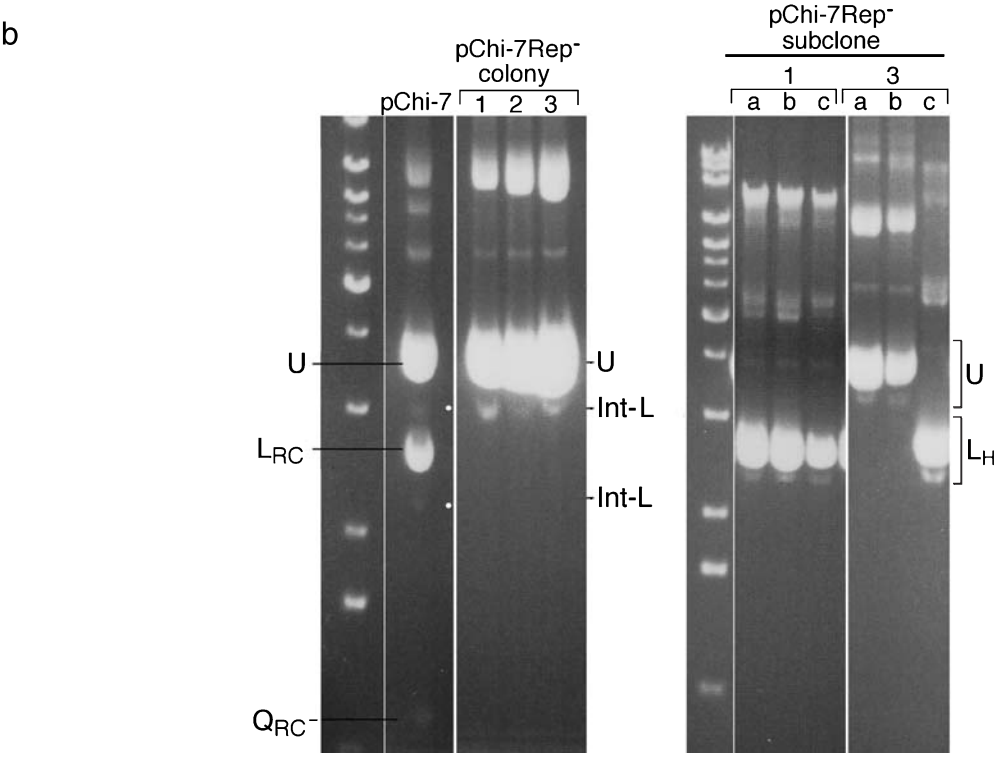
includes a group of diverse animal viruses with small, closed-circular, single-stranded (ss) DNA [19, 22]. Two genotypes of PCV have been identified: PCV type 1 (PCV1) and PCV type 2 (PCV2), and their genomes share 68–76% nucleotide (nt) sequence homology [17]. Recent works showed that PCV2 can be further separated into two genotypic subgroups [8, 20]. PCV has an ambisense circular genome that encodes proteins both by the encapsidated viral DNA, and by the complementary DNA of the double-stranded (ds) replication intermediate synthesized in the host. Two coding regions of opposite polarity, the Rep gene on the right and the capsid gene (Cap) on the left, are separated at their 5′-ends by the origin of DNA replication (Ori) intergenic region (IR) of approximately 80 nucleotides (Fig. 1). Sequence and structural motif similarities indicate that PCV replicates its genome via a rolling-circle replication (RCR) mechanism in a manner similar to that of members of the family *Geminiviridae* (see review in [14, 15, 21]) or the family *Nanoviridae* (see review in [13]) with modifications at the Ori proposed by the RCR ''melting-pot'' model [4, 5].

Engineering head-to-tail (HTT) direct tandem constructs of PCV has become a popular practice to generate infectious viruses or vaccine candidates against PCV-associated diseases [10–12, 24]. Two mechanisms, RCR and homologous recombination, have been proposed to account for the excision and generation of unit-length viral genomes from HTT constructs [23, 26]. In this work, the products derived from RCR are indicated by subscript ($_{-RC}$) and the products derived from homologous recombination are indicated by subscript ($_{-H}$). Recently, we demonstrated that HTT constructs (pChi-6 and pChi-7) containing 1.75 copies of PCV1/PCV2 DNA (0.8 copy of PCV1 and 0.95 copy of PCV2) with two PCV Oris inserted into the pBluescript SK^+ (pSK^+) bacterial plasmid (Stratagene, La Jolla, CA) with the colicin E_1 ($ColE_1$) Ori yielded three different molecular-size DNA species when transformed into *Escherichia coli (E. coli)*: the input construct (*U*), a unit-length chimeric $PCV1_{Rep}/PCV2_{Cap}$ genome with a composite Ori (Ori-$_{2L/1R}$) but lacking the plasmid vector (Q_{RC}), and a molecule consisting of the remaining 0.75 copy $PCV1_{Cap}/PCV2_{Rep}$ genome with a different composite Ori (Ori-$_{1L/2R}$) together with the bacterial plasmid (L_{RC}) [7] (Fig. 1). Replication of *U* was presumably via the unidirectional theta-replication mechanism utilizing the $ColE_1$ Ori [16, 27] involving bacterial proteins only, while characteristics of the other two DNA species (Q_{RC} and L_{RC}), including a requirement for two PCV Oris and the virus-encoded replication initiator Rep protein, suggested they were generated via the RCR copy-release mechanism. Interestingly, two PCV-encoded initiator proteins, Rep and Rep′ are essential for PCV DNA replication in mammalian cells, but only the Rep protein is required in *E. coli* [7]. For the generation of Q_{RC}, DNA synthesis initiates at the PCV1 Ori-$_{1R}$ and terminates at the PCV2 Ori-$_{2L}$. During initiation, the PCV Rep protein is necessary for cleaving the PCV1 Oc8 sequence ($A_1G_2T_3A_4T_5T_6\downarrow A_7C_8$, which contains the nick site indicated by ↓) [25] between T_6 and A_7 to generate a 3′-OH end for leading-strand DNA synthesis, and the polymerization process is carried out by the bacterial replication machinery. During termination, Rep nicks the PCV2 Oc8 sequence and then reconstitutes a chimeric Ori-$_{2L/1R}$ by joining the ends of the displaced chimeric unit-length $PCV1_{Rep}/PCV2_{Cap}$ genome and releasing the circular ss-Q_{RC} molecule. For the generation of ss-L_{RC}, DNA synthesis initiates at the PCV2 Ori-$_{2R}$ and terminates at the PCV1 Ori-$_{1L}$. Both ss-Q_{RC} and ss-L_{RC} are then converted to their respective ds species. Any L_{RC} or Q_{RC} molecule containing a chimeric PCV1/PCV2 Ori (Ori-$_{2L/1R}$ or Ori-$_{1L/2R}$) would indicate that the Rep protein was involved in its generation. Recent work with the HTT construct also showed that a stem-loop structure at the Ori of PCV is essential for termination, but not initiation, of RCR [9].

In this work, two previously engineered heterologous PCV1/PCV2 HTT tandem constructs, pChi-7Rep$^-$ (Fig. 2) [7] and pB (Fig. 3) [9], were utilized to examine whether homologous recombination contributes to the excision of unit-length Q_H from *U* to produce L_H in *E. coli*. Similar to previous work, heterologous tandem repeats were employed because the components essential for PCV1 or PCV2 DNA replication (the Ori-IR and the Rep-complex) are interchangeable [6, 18]. Furthermore,

the nucleotide differences between the two viral genomes are easily discernable and permit easier identification of the mechanisms used for generating various *L* and *Q* DNA species. We reasoned that the L_H and Q_H DNA species produced via homologous recombination would be different from the



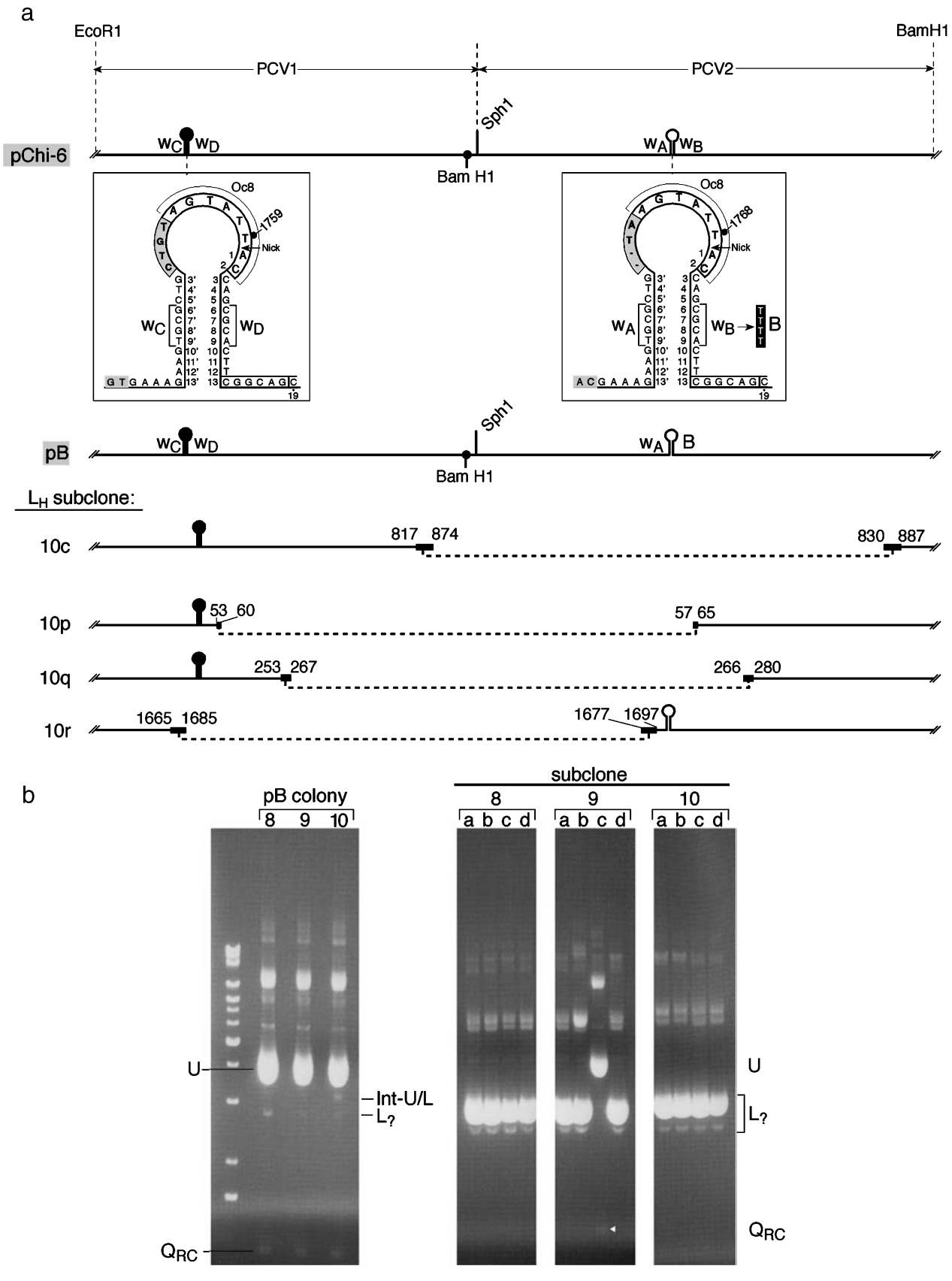


L_{RC} and Q_{RC} molecules generated via RCR. The L_{RC} and Q_{RC} molecules would always initiate and terminate within the Oc8 motif and have a chimeric Ori (Ori-$_{1L/2R}$ or Ori-$_{2R/1L}$) composed of both PCV1 and PCV2 sequences (C-B stem-loop sequence for L_{RC} and A–D stem-loop sequence for Q_{RC}) (Fig. 1). In contrast, homologous recombination might be expected to occur at various sites throughout the tandem construct and would yield L_H and Q_H molecules that contain an Ori (Ori-$_{1L/1R}$ or Ori-$_{2L/2R}$) with either, but not both, PCV1 or PCV2 nucleotide sequences.

We first investigated whether homologous recombination can occur in the absence of RCR with construct pChi-7Rep$^-$, which does not contain a functional Rep gene (Fig. 2). Briefly, early termination codons (previously shown to render PCV1 or PCV2 genomes non-replicative when transfected into mammalian PK15 cells) [1–3] were introduced into pChi-7. These mutations were engineered into both the PCV1 *Rep* (nt 727) and the PCV2 *Rep* (nt 740) sequences to generate pChi-7Rep$^-$ (Fig. 2a). In the absence of the Rep protein, it is expected that RCR would not take place and thus neither L_{RC} nor Q_{RC} would be synthesized. After transformation of the construct into TOP10 competent cells (Invitrogen, Carlsbad, CA), plasmid DNAs were isolated from the overnight bacterial cultures using a miniprep plasmid DNA kit (Promega, Madison, WI), and electrophoresis (2.5 μg of DNA) was carried out in a 1% agarose gel. With the parent pChi-7 construct, three previously described species (*U*, L_{RC} and Q_{RC}) [7] and two faint DNA species that migrated to an intermediate (Int-) position between *U* and L_{RC} (*Int-U/L*) and slightly ahead of L_{RC} (*Int-L*) were observed (Fig. 2b). Upon re-transformation and sequencing, (*Int-U/L*) yielded (*U* and L_{RC}) and (*Int-L*) yielded (L_{RC}). Thus, (*Int-U/L*) and (*Int-L*) are DNA replicative intermediates. As expected with the mutated pChi-7Rep$^-$ construct, L_{RC} and Q_{RC} were not detected. However, a DNA species migrating to an intermediate location between *U* and *L* (labeled *Int-U/L*) was detected in 2 of 3 bacterial colonies analyzed. DNAs from colony 1 and from colony 3 were purified from the gel individually and re-transformed into bacteria. Three ampicillin-resistant subclones were picked from each transformation and analyzed. Whereas the DNA from subclones 1a, 1b, 1c and 3c migrated to the *L* location, subclone-3a and -3b DNA migrated to the *U* location. Sequence analysis showed that all 4 subclone *L* DNAs contained only PCV2 Ori sequences, which indicates that they were L_H molecules generated via homologous recombination (Fig. 2a). Thus, the *Int-U/L* DNA species from colony 1 and 3 were DNA replicative intermediates. All three subclones of colony 1 (*a* to *c*) were identical, and the cross-over point of these L_H molecules was located within a 44-nt homologous sequence between nt 470–513 of PCV1 and nt 483–526 of PCV2. The cross-over point of the subclone 3c L_H molecule was within a 26-nt homologous sequence between nt 704–730 of PCV1 and nt 717–743 of PCV2. Further examination (isolation, re-transformation and sequencing) of the minor DNA species that migrated slightly ahead of the main L_H DNA species showed that they were L_H replicative intermediates.

We next examined whether homologous recombination can occur in the presence of RCR with construct pB (a derivative of pChi-6), in which the wild-type (w) sequence at position B (w_B) within the PCV2 stem-loop sequence was replaced by 4 T nucleotides (Fig. 3). It was expected that the com-

Fig. 2. Identification of homologous-recombination-derived L_H DNA species from the pChi-7Rep$^-$ construct in *E. coli*. **a** Schematic representation of pChi-7Rep$^-$ or pChi-7 that contain mutations in both Rep genes. The open reading frame (ORF) nucleotide coordinates for capsid, Rep and Rep' are indicated below each gene. The L_H DNA species generated via homologous recombination from colonies 1 and 3 are indicated. The common nucleotides where cross-over (indicated by dotted lines) took place are denoted by black boxes. **b** Agarose gel electrophoresis of plasmid DNAs recovered from *E. coli* TOP10 cells after transformation. The DNA constructs used in each experiment are indicated at the top of each lane. The predominant closed-circular double-stranded molecules are labeled *U*, L_{RC} and Q_{RC}. The minor DNA species that migrated to an intermediate position between *U* and *L* is labeled *Int-U/L*, and the minor DNA species that migrated faster than *L* is labeled *Int-L*. Subclone-1 (*a* to *c*) and subclone-3 (*a* to *c*) DNAs represent plasmids recovered from re-transformation of pChi-7Rep$^-$ colony-1 and colony-3 DNAs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10c
817 874
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8 9 10
a b c d
U
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Q_RC

plementary wild-type w_Aw_D stem-loop would yield Q_{RC}, while the mis-matched w_CB stem-loop would fail to generate L_{RC} via the RCR mechanism. The results (similar to a previous report [9]) showed that Q_{RC} containing the chimeric Ori-$_{2L/1R}$ with the w_Aw_D stem-loop and an unexpected *L*-migrating DNA species (labeled $L_?$ prior to sequence identification) were observed (Fig. 3b). Sequence analysis showed that $L_?$ contained two variants, vL and cL. Both vL and cL contained a PCV1/PCV2 chimeric Ori, which is indicative of appropriate nicking and joining of Oc8 of PCV1 and PCV2 by the Rep protein during their generation. One possible scenario for the generation of vL containing the chimeric Ori-$_{1L/2R}$ with mis-matched w_CB stem-loop sequence is that when both the PCV1 and PCV2 Ori were nicked during replication, the 3′-end of PCV1 Ori was ligated to the 5′-end of PCV2 Ori and thus retained the mutated 4 T nucleotides at position B. The vL species was then presumably propagated via the theta-replication mechanism utilizing the $ColE_1$ Ori of pSK^+. For the generation of cL containing the chimeric Ori-$_{1L/2R}$ with corrected wild-type w_Cw_B stem-loop sequence, template-strand switching [4, 5] must have taken place to correct the mismatched w_CB sequence of vL during DNA replication. Examination of 10 re-transformed colonies of clone 8-$L_?$ showed that 8 of 10 colonies yielded vL, while 2 of 10 colonies yielded cL. In any event, the generation of vL or cL required a functional Rep protein, which indicates involvement of the RCR mechanism.

The pB HTT construct also yielded a minor *Int-U/L* species that migrated to a location intermediate between *U* and *L* in colonies 8, 9 and 10 (Fig. 3). The *Int-U/L* DNA from each colony was purified from the gel and re-transformed into bacteria. Four subclones were picked from each transformation and analyzed. Subclone 9c yielded *U* and a very faint Q_{RC} (after sequence determination), but no *L*-migrating DNA species, while the other 11 recovered DNAs exhibited only *L*-migrating DNA (labeled $L_?$ prior to sequence identification). Sequence analysis showed that 10 of 11 $L_?$ subclones (with the exception of subclone 10c) contained the chimeric Ori-$_{1L/2R}$ with mis-matched w_CB stem-loop sequence, which indicates that they were vL DNA molecules. Subclone 10c contained only a PCV1 Ori, which indicates that it is a L_H species generated via homologous recombination. The cross-over point of subclone 10c lies within a 58-nt homologous sequence between nt 817–874 of PCV1 and nt 830–887 of PCV2. Further examination (isolation, re-transformation and sequencing) of the minor DNA species that migrated slightly ahead of the main $L_?$ DNA showed that they were replicative intermediates of their respective *L*-migrating DNA species.

The presence of L_H among the subclone-10c DNA (1 of 4 clones) suggested that there could be other L_H molecules present among the *L*-migrating DNAs of colony-10. Thus, the *L*-migrating DNA ($L_?$) of colony 10 was analyzed after re-transformation into bacteria. Sixty-nine individual bacterial colonies were picked and sequenced. The results showed that 63 subclones yielded vL DNA containing the chimeric Ori-$_{1L/2R}$ with the mismatched w_CB "stem-loop" sequence, 3 subclones yielded the corrected cL DNA containing the chimeric Ori-$_{1L/2R}$ with wild-type stem-loop sequence, and the remaining 3 subclones (suclones 10p, 10q and 10r) contained either a PCV1 or a PCV2 Ori, indicative of being generated via homologous re-

Fig. 3. Identification of homologous-recombination-derived L_H DNA species from the pB construct in *E. coli*. **a** Schematic representation of the pB construct, which contains a 4-T nucleotide mutation in position B of the PCV2 stem-loop. The stem-loop nucleotide sequences of PCV1 and PCV2 are indicated in boxes. The common nucleotides where cross-over (indicated by dotted lines) took place to generate each L_H molecule are denoted by black boxes. **b** Agarose gel electrophoresis of plasmid DNAs recovered from *E. coli* TOP10 cells after transformation with pB. The predominant closed-circular double-stranded molecules are labeled *U* and Q_{RC}. The minor DNA species that migrated to an intermediate position between *U* and *L* is labeled *Int-U/L*. The DNA species labeled $L_?$ indicates its position in the gel prior to nucleotide sequence identification. Subclone-8 (*a* to *d*), subclone-9 (*a* to *d*) and subclone-10 (*a* to *d*) DNAs represent plasmids recovered from re-transformation of pB colony-8, colony-9 and colony-10 DNAs, respectively

combination (Fig. 3). The L_H DNAs of subclones 10p and 10q contained a PCV1 Ori, while subclone 10r contained a PCV2 Ori. Sequence analysis showed that the cross-over point of subclone 10p lies within an 8-nt homologous sequence between nt 53–60 of PCV1 and nt 57–65 of PCV2, the cross-over point of subclone 10q lies within a 14-nt homologous sequence between nt 253–267 of PCV1 and nt 266–280 of PCV2, and the cross-over point of subclone 10r lies within a 21-nt homologous sequence between nt 1665–1685 of PCV1 and nt 1677–1697 of PCV2.

In this work, we present evidence that the excision of a unit-length viral genome from a PCV HTT construct via homologous recombination can occur in the presence or absence of RCR in *E. coli*. The cross-over point for the generation of L_H was invariably traced to a stretch of common nucleotides of variable length between PCV1 and PCV2, and the L_H molecules generated may retain either the PCV1 or the PCV2 Ori. The amount of L_H produced in each of the examples analyzed was quite low. In comparison with the parent pChi-7 construct that yielded an observable amount of L_{RC} and Q_{RC}, the mutated pChi-7Rep$^-$ construct generated little, if any, *L*-migrating DNA via homologous recombination. Sequence analysis of the $L_?$ DNAs of pB showed that 3 (subclones 10p, 10q and 10r) out of 69 subclones and 1 (subclone 10c) out of 11 subclones were generated via homologous recombination. Furthermore, previous work showed that *Q*-migrating DNAs containing either PCV1 or PCV2 Ori (i.e., Q_H) were not detected among one hundred pChi-6 Q_{RC} molecules analyzed [7]. Taken together, these result suggest that RCR plays a dominant role in the excision of the unit-length viral Q_{RC} genome from HTT constructs to generate L_{RC}, and homologous recombination plays a relatively minor role in the excision of Q_H from identical HTT constructs for generating L_H in *E. coli*.

Acknowledgments

The author thanks S. Pohl, Dr. D. Alt and K. Halloum for technical assistance, and M. Marti and S. Ohlendorf for manuscript preparation.

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